THE RELATIONSHIP BETWEEN HUMAN ERYTHROCYTE LIPOPOLYSACCHARIDE RECEPTOR AND AN INHIBITOR OF COMPLEMENT MEDIATED LYSIS

by

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A DISSERTATION PRESENTED TO THE
GRADUATE COUNCIL OF THE UNIVERSITY OF FLORIDA
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA
1978

LEDGMENTS

I wish to express sincere appreciation for [illegible] criticisms and patiant guidance given me by Dr Edward M Hoffmann

I also would like to extend thanks to Dr L O Ingram, Dr A S Bleiweis, Dr Paul W Chun, Dr Lester W Clem, Dr Paul Smith and Mr Jim Milam for supplying constant help and encouragement

To all of tha members of my laboratory and departmental familiy ovar the last four and a half years, I offer my sincere gratitude for their constant encouragement, patience, caring and all of tha happy times we va shared

I wish also to add my thanks to Mrs Sandy Canneila for har encouragement and her excellant and dadicated assistance in the typing of this manuscript

Finally, but most important of all, for their continual prayers, halp, guidance, unfailing iove and for always being thare through the good and hard times, I thank my parents Mr. and Mrs Charles and Sarah McKnight and my ioving son Jacques Without thase this would never hava been possible.

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SUMMARY OF ABBREVIATIONS

C	complement
C1, C2, C3, C4, C5, C6, C7, C8, C9[1]	complement components
E	erythrocyte
A	antibody
HA	hemagglutination
HAI	hemagglutination inhibition
EDTA	ethylenediamine tetracetate

[1]The nomenclature of complement used conforms to that proposed as a result of a series of World Health Organizations (Immunochemistry, 7:137, 1970)

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By

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December, 1978

Chairman: Edward M. Hoffmann
Major Department: Microbiology and Cell Science

Human erythrocyte membrane extracts with receptor specificity for the lipopolysaccharides of gram negative bacteria were found to be rich in IH, a complement inhibitory substance originally isolated and described from human red cell membranes. The possibility that the LPS-receptor and IH inhibitor might be the same macromolecule was considered.

In this investigation it was demonstrated that although closely associated, the two activities are biologically distinct and separable. This conclusion was supported by the results of five experimental approaches. Small, but distinct differences were observed in the elution profiles of the two activities when crude extracts of erythrocyte membranes were subjected to sepharose 4B, DEAE-Sephadex chromatography and polyacrylamide disc gel electrophoresis. Complete separation of the two activities was accomplished by a shift in the pH of the membrane extraction conditions. Further, differences between the two activities based on their ability to spontaneously bind to sheep erythrocytes were

demonstrated [illegible] presented indicating that the membranes of sheep erythrocyte[illegible] consist of molecules with LPS-receptor specificity but were devoid of IH inhibitor activity

Additional studies indicated that LPS bound to cell membranes can activate either the alternative or classical complement pathways and that IH inhibitor associated with cell membranes can block LPS-induced complement lysis of red cells despite the fact that complement activation has occurred

INTRODUCTION

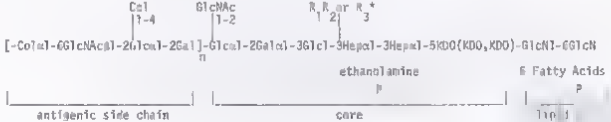
The endotoxins or lipopolysaccharides (LPS) of gram negative bacteria are among numerous antigens known to be capable of fixing to the membranes of erythrocytes and other mammalian cells *in vitro* and under certain conditions, *in vivo* (1). Because of their unique ability to modulate the immune response in a wide variety of ways, they have emerged as a complex and fascinating class of macromolecules. Functionally, endotoxins have been shown to have many different properties. Due to their chemical makeup and localization in the outer membrane of the bacterial cell envelope they have been shown to play a major role in the establishment of a selective permeability barrier (2,3) and in serving as receptors for certain bacteriophages (4).

Of interest to the immunochemist, however, is the fact that interaction of LPS with components of the immune system may lead to a single or combination of physiological responses. These include: toxicity, mitogenicity, immunogenicity, tolerance and activation of complement. Although much is known concerning the general nature of these responses, the mechanisms of the cell associated events responsible for their development in the presence of LPS are still not fully understood.

A great deal of information about the chemical structure of LPS, from a variety of organisms, has accumulated. Although it has been recently recognized that LPS isolated from a given organism is

heterogenous (5). LPS of most gram negative wildtype organisms appear to share the same basic molecular composition As illustrated in Figure 1, all consist of three regions The first region, the O-specific polysaccharide antigen which is made up of repeating units of five to eight monosaccharides, carries the main serologic specificity for a given organism Numerous serological groups differing in O-antigen specificity are now recognized and the polysaccharides accordingly show wide inter- and intrageneric variations in composition (6) Of interest is the fact that natural antibodies to this region are found in most animal species but do not always appear to be protective, and in some cases a lethal gram negative bacteremia develops despite high titers of O-specific antibody (7) Region two consists of a short outer core which contains glucose (glc), galactose (gal), and N-acetylglucosamine (Glc NAC), and an inner core of L-glycero-D-mannoheptose, phosphate, ethanolamine and three molecules of 3-deoxyoctonate (KDO) Unique to the endotoxins of gram negatives, 3-deoxyoctonate provides a linkage site to the third region, the lipid A moiety Lipid A is basically composed of a phosphorylated glucosamine backbone to which are attached fatty acids and ethanolamine residues The nature and distribution of the lipid A fatty acids varies among bacterial groups with the inner core polysaccharide composition remaining constant (7) The complete LPS containing O-specific antigen is designated smooth (S), and all mutants lacking O-specific side chains are referred to as rough or R-forms

Many early studies concerned with the interaction of endotoxins and biological systems were carried out using either whole bacteria



LPS1-n=11
LPS11-n=3.

*R_1 = O, R_2 = Glc-4 GlcNh$_2$, R_3=Hep

Figure 1. Probable structure of LPS of Escherichia coli. There are probably three separate core structures present with R as shown for each. Pyrophosphate bridges probably cross link adjacent lipid molecules yielding at least trimers. Abbreviations: Col, colitose; GlcNAc, N-acetylglucosamine, Glc, glucose, Gal, galactose, GlcN, glucosamine, Hep, heptose, and KDO, 2 keto-3-deoxyoctanoci acid. A protein covalently linked to LPS may also be present (6)

or sterile heat-killed LPS preparations in the fluid phase. This was in spite of the fact that it had been shown that heated LPS (100°C for 60 minutes) could be coated onto the surface of a number of cells including erythrocytes (8). The latter are often the model target cells for hemolytic assays. It has become increasingly clear that the biological consequence of either an in vivo or in vitro encounter with LPS, whether cell bound or partially purified, is dependent upon its fixation to various target cells (9). For example, it was reported that patients suffering from endotoxin shock and sepsis due to gram negative bacteria had greatly reduced levels of blood platelets and that the platelets contained LPS. It has further been established that human platelets possess an endotoxin binding receptor, which when triggered by interaction with LPS, results in the release of a vaso-active amine, 5-hydroxytryptamine (5-HT) and the unmasking of clot-promoting activity (10). In animal studies, it was observed that guinea pigs injected intravenously with LPS also show a 95% decrease in blood platelets with a concomitant shortening of the clotting time (11). Additionally, it has been reported that there is a direct relationship between the susceptibility of different strains of mice to the lethal effects of Salmonella endotoxin and the affinity of their red cells for either heat-killed Salmonellae or free LPS (12).

Little was known about the nature of the attachment of LPS to any cell until the late sixties, when G. Springer isolated an extract from the membranes of human erythrocytes having a high affinity and specificity for the lipopolysaccharides of a variety of gram negative

bacteria (13-15) This material, designated as an LPS-receptor, has now been purified to homogeneity and characterized Springer has reported that the LPS-receptor is a lipoglycoprotein, rich in N-acetylneuraminic acid (NANA), galactose, hexosamine and contains about 61% protein (16). It appears to be a pentameric molecule with a molecular weight of about 228,000 daltons The LPS-receptor functions by direct interaction with groups on the LPS molecule which provide an attachment site for tissue components (16) Strong evidence has accumulated suggesting that this attachment site is the lipid A moiety of LPS (17) This high affinity of the LPS-receptor for endotoxins is quite remarkable because both macromolecules are highly negatively charged the receptor, because of its high NANA content and LPS because of its phosphoric acid radicals.

Because the immunological specificity of LPS bound to erythrocytes remains unchanged, Springer has suggested that the lipid A of LPS binds to the specific receptor via clusters of hydrophobic amino acids which makeup about 40% of the total peptide content of the receptor leaving the polysaccharide available for the reaction with antibodies (16) A complete understanding of the orientation of LPS on tissues, bound either by specific receptors or by non-specific mechanisms, may come from studies involving the interaction of cell bound LPS with serum complement

The anticomplementary effects of LPS have long been established For some time, evidence seemed to suggest that the single most important factor in the development of a noxious response to endotoxins was the direct interaction of the lipid A region with biological

systems including the complement system (18) Whatever the mechanism, mounting evidence seems to implicate complement as a mediator of a number of the deleterious responses to endotoxins in experimental animals and man (8)... More importantly, recent evidence indicates that the ability of LPS to initiate a complement response is not confined to the lipid A moiety but appears to involve the polysaccharide core as well (18)

It has become increasingly clear that a major role of the complement system during an immune response is the mediation of enhanced phagocytosis with a concomitant induction of an inflammatory response... This is accomplished by the sequential activation of the proteins which make up the complement system The activation process can be divided into three major stages a recognition stage, the generation of C3 cleavage enzymes and C3 activation stage, and a terminal or membrane attack stage (20-22) Under normal conditions, complement proteins exist in the serum as inactive precursors, and are activated by either of two pathways -- the classical or alternative The components of these pathways and their reaction requirements are summarized in Table 1 The two share a number of similar characteristics but differ, basically, in the reactants and the sequence of reactions of the first two stages

The capacity of endotoxins to activate the complement system by a mechanism which requires neither antibodies to LPS nor the participation of the early complement components was demonstrated more than twenty years ago (23) More recently, it was recognized that fluid phase activation of complement by LPS is not restricted to the

TABLE I

Comparative Properties of the Classical and Alternative Complement Pathways

		Classical	Alternative
Activating agents			
Immunoglobulins of	human	IgG1,IgG2, IgG3,IgM	IgA and IgE
	rabbit	IgG	IgM
	guinea pig	IgG2	IgG1
	ruminant	IgG1	IgG2
Miscellaneous		(Lipid A)	Inulin, Zymosan, Endotoxin or LPS, CVF
Activating site		Fc fragment	F(ab')2 (rabbit) or Fc fragment (human)
Factors required to generate C3 convertase		C1[a]	Properdin, factor D
		C4	C3b
		C2	factor B
Total serum requirement		Dilute	Concentrated
Divalent cation requirements		Ca and Mg	Mg

[a]C1 is a trimolecular complex of C1q, C1r and C1s. Classical pathway activation is initiated when C1q binds to immunoglobulin.

[illegible] pathway [illegible] of LPS have been demonstrated to activate the classical pathway in either the presence or absence of specific antibodies (8,24-26,27)

Normally, during classical pathway activation, the recognition stage is initiated by the fixation of the first component of the complement system, C1, to IgG or IgM immunoglobulins of specific immune complexes. This fixation and activation of C1 generates a C1 esterase whose major substrates are the fourth and second components of the complement system.

Endotoxins in the fluid phase have been shown to initiate the activation of the complement system in vitro as described above in the presence of predominantly IgM antibodies directed against the O-polysaccharide antigen (28). Unique to the endotoxins of certain gram negatives, however, is the capacity of the lipid A moiety of the molecule to fix C1 directly resulting in a antibody independent initiation of the classical complement cascade (8,29).

Alternative pathway activation, on the other hand, is triggered by direct activation of an initiation factor (IF) by endotoxins, or any one of a number of naturally occurring polysaccharides, and aggregates of IgA or IgE (30). It has been established that alternative pathway activation of the complement system involves the core polysaccharide region of the LPS molecule (31). Additionally, it has been shown that the length of the O-specific polysaccharide antigen and differences in the carbohydrate content may also play a role in this mechanism of activation (8).

In stage two of the classical pathway activation scheme, C3 convertase is generated when C1 esterase cleaves C4 and C2 in the presence of magnesium yielding the cell bound C4b2a enzyme. This convertase cleaves the third component of complement (C3) into two fragments, C3a and C3b. The latter fragment is unstable and has a highly reactive hydrophobic binding site. As a result of binding to target membranes at sites adjacent to the membrane bound C3 convertase, a new enzyme C5 convertase, whose major substrate becomes the fifth component of complement, is generated. In addition, C3b undergoes secondary changes giving rise to an immune-adherence site capable of binding to a variety of effector cells of the immune system which bear specific C3b receptors (32). Additionally, C3b, either bound hydrophobically to a cell surface or free in solution, can produce further splitting of C3 via the C3 feedback cycle of the alternative pathway described below. It is this amplification of the generation of C3b and its deposition via immune adherence onto the surface of specific target cells (and in some cases innocent bystanders), where the major function of the complement system is realized.

The mechanism of the alternative pathway generation of the C3 convertase is a bit more complicated in that there is a direct requirement for preformed C3b, the source of which is still not fully understood. This C3b in combination with factor B plus properdin and factor D forms the alternative pathway convertase "Factor BbC3b" capable of splitting C3 into C3a and C3b (33). This splits more native C3 giving rise to further C3b and the cycle is again repeated. This reaction

is magnesium dependent and unlike classical pathway activation it is inhibited by high concentrations of calcium (34) Under proper experimental conditions these cation requirements make serum chelated with either ethylenediamine tetraacetic acid (EDTA) or ethyleneglycol tetraacetic acid (EGTA) useful reagents for distinguishing between the two pathways (35) The former, being an effective chelator of both calcium and magnesium blocks the activation of the two pathways, while the latter, a less effective chelator of magnesium, preferentially blocks the classical pathway

The last and final stage of complement activation is the same for both pathways and is initiated with the cleavage of C5 into two fragments, C5a and C5b by the C5 convertase The larger C5b fragment then reacts sequentially with C6 and C7 to form either a cell bound or fluid phase trimolecular complex $\overline{C5b67}$ The cell bound complex has the capacity to bind C8 and C9 If the cell to which this C5-C9 complex is associated is sensitive to complement mediated cytolysis, lysis ensues (36)

Fluid phase $\overline{C5b67}$, C3a, and C5a are potent anaphylatoxins and chemotactins (37,38) Once leukocytes such as polymorphonuclear leukocytes (PMN) and macrophages have migrated to the site of complement activation, phagocytoses is initiated As previously stated, the phagocytic process is enhanced by the fixation of complement components, especially C3b, onto the surface of particulate antigens or target tissues which promote adherence, thus facilitating ingestion (39) Release of liposomal hydrolases, either as a direct consequence of ingestion or expulsion of an indigestible target into the surrounding tissues, results in the generation of additional chemotactic factors (40,41) This

[illegible] stage in the development of an inflammatory response

Severe tissue damage resulting from unchecked amplification of the activation of the complement system is probably minimized by the presence of two known serum inhibitors, C1 esterase inhibitor (42) and conglutinin activating factor (KAF) also known as C3b inactivator (43). Perhaps there are other humoral or cellular inhibitory factors still to be defined.

Many of the studies cited here employed fluid phase LPS for *in vitro* assays. Neter was the first to demonstrate that LPS coated onto the surface of sheep erythrocytes were sensitive to lysis by an antibody dependent classical complement pathway mechanism (44). Phillips and Morgenhegen employing sheep erythrocytes treated with LPS extracted by the Westphal-phenol procedure (45) confirmed Neter's observation showing the need for the presence of a naturally occurring γ2 globulin for complement activation with a sparing consumption of the early components C1-C4 (46).

The *in vivo* consequences of the intravenous administration of endotoxins to normal and complement deficient animals have been studied. Both complement pathways are activated with the classical pathway being required for the development of many of the pathophysiological symptoms such as the thrombocytopenia observed in many experimental endotoxin infections (11). Some investigators have suggested that classical pathway activation due to an antibody mediated fixation of LPS to various cells such as platelets and erythrocytes is more effective in

cell membranes
release of
(47,48) Alternative pathway activation has not been shown to mediate the release of membrane damaging factors (49) In vivo systems are difficult to evaluate because of the large number of parameters which must be considered Therefore, much study is needed before a complete understanding of the mechanism involved can be obtained

Regardless of the nature of the system (in vitro or In vivo) or the state of the endotoxin (free or cell bound), the interaction of LPS with the complement system in the presence of specific antibody is extremely efficient, giving full response with sparing consumption of antibody and C1-C4 (48) Virulence of gram negative bacteria would then appear to be related to the fate and site of free endotoxins released as a direct consequence of cell death due to phagocytosis and interaction of this free endotoxins with other cell systems in the presence of serum factors

As previously stated, erythrocytes and now other cells have been shown to have membrane receptors which bind endotoxins The biological role of these receptors is still not clear, however, fixation of LPS to the cell does appear to be a precondition for the triggering of many incitements of the immune system by LPS (49)

Hoffmann, in the late 1960s, isolated and described extracts from the membranes of human erythrocytes capable of inhibiting the hemolytic activity of complement when sensitized sheep erythrocytes were used as target cells (50) Extracts isolated at two different lonicities

[illegible] different binding affinities for the [illegible] of sheep erythrocytes. Material prepared at an ionic strength of 0.11 was shown to be capable of binding to sheep erythrocytes and protecting them from immune lysis, and has been designated IH inhibitor

An extract prepared under the same conditions but at a lower ionicity was incapable of binding to sheep erythrocytes but was capable of accelerating the decay of the complement component intermediate $\overline{EAC142}$ to $\overline{EAC14}$ This material was designated DAF for decay accelerating factor (51)

Preliminary studies indicate that the IH inhibitor is a large molecule with a molecular weight greater than 250,000 It appears to contain at least two sugar moieties, glucose and galactose and it is about 4% protein by weight (50)

Existing data suggest that the IH inhibitor probably acts at the C3 convertase step This was suggested by the finding that IH coated sheep erythrocytes in the intermediate state $\overline{EAC142}$ consumed less C3 than untreated controls and that the inhibitory effects of IH ceased once activated C3 became fixed to the cell bound C3 convertase (51,52)

Attempts to more fully define the biochemical and biological properties of these macromolecules have been hampered by the inability to obtain them in a highly purified state

Erythrocytes of different species differ in their susceptibilities as target cells in immune hemolysis, with sheep and chicken erythrocytes being far more sensitive than human and guinea pig erythrocytes Differences within the same species have also been observed (53) For

[illegible] been reported that patients suffering [illegible] paroxysmal [illegible] nocturnal hemoglobinuria (PNH) have at least two populations of erythrocytes based on their susceptibility to immune hemolysis (54), with at least one subpopulation exhibiting extreme sensitivity to attack by an antibody-independent complement mediated mechanism. It has been demonstrated that extracts from the erythrocyte stromata of these patients have reduced levels of IH inhibitor activity.[1] Additionally, evidence has been presented which suggests that there is a parallel between the presence of DAF on the membranes of the erythrocytes of certain species and resistance to the cytolytic effects of complement (53).

A comparison of the isolation schemes for preparing the IH inhibitor and the LPS-receptor revealed a marked similarity between the two. Both activities are confined to that fraction of the membrane extractable by a butanol-water mixture at 4°C and at an ionic strength of 0.15 or below. All of the activity of either material appeared to be localized only in the aqueous layer of the butanol extracted membranes. Ion exchange chromatography of extracts containing either the LPS-receptor or IH inhibitor activities indicate that both are eluted under identical conditions. These observations would suggest that the two activities may be similar or even identical.

The principle objective of this investigation was to determine if the IH inhibitor and LPS-receptor are either the same or closely

[1]Data supported by personal experiments

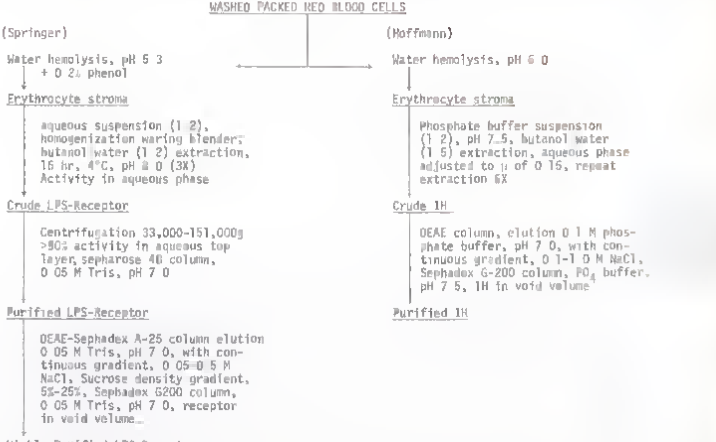
related Although it has been observed that human erythrocytes are highly resistant to LPS mediated immune hemolysis, the reason for the refractoriness of these treated erythrocytes has not been defined Sheep erythrocytes, as previously stated, are normally sensitive to immune lysis but may be rendered resistant by treatment with diluted extracts of the partially purified IH inhibitor Therefore, the second objective of this investigation was to explore the biological consequences of the IH inhibitor in the interaction of LPS treated erythrocytes and serum complement, in an attempt to clearly establish a biological role for the IH inhibitor

MATERIALS AND METHODS

Erythrocytes Out-dated human blood (group O, Rh positive) containing citrate-phosphate-dextrose as anticoagulant was obtained from the Civitan Regional Blood Center (Gainesville, FL) Whole sheep blood was taken by venipuncture from animals maintained at the Animal Research Laboratory of the J. Hillis Miller Health Center (Gainesville, FL) One volume of blood was mixed with an equal volume of sterile modified Alsever's solution (55) and the blood was stored at 4°C for up to one month

Preparation of erythrocyte stromata Human and sheep erythrocyte stromata were prepared by the method of Springer et al (56) Erythrocytes from whole blood were pelleted at 4°C by centrifugation for ten minutes at 500xg and the plasma and buffy coat were discarded The packed cells were washed three times with phosphate buffered saline (0 13 M sodium chloride plus 0 005 M potassium phosphate) at pH 7 4 and lysed in 10 volumes of distilled water at 4°C In the initial studies, the pH was adjusted to 5 3 with acetic acid and phenol was added to a final volume of 0 2% The stromata were allowed to settle overnight at 4°C and the supernatant fluid was removed Ten volumes of cold distilled water were added, and the pH was readjusted to 5 3 The stromata were sedimented either by settling or centrifugation and the entire procedure was repeated six times with the addition of phenol

Figure 2 A schematic representation of the extraction and purification procedures that were employed in the preparation of the LPS receptor of Springer and the IH inhibitor of Hoffmann


WASHED PACKED RED BLOOD CELLS
(Springer)
(Hoffmann)
Water hemolysis, pH 5 3
+ 0 2% phenol
Water hemolysis, pH 6 0
Erythrocyte stroma
Erythrocyte stroma
aqueous suspension (1 2), homogenization waring blender, butanol water (1 2) extraction, 16 hr, 4°C, pH 8 0 (3X) Activity in aqueous phase
Phosphate buffer suspension (1 2), pH 7 5, butanol water (1 5) extraction, aqueous phase adjusted to pH of 0 15, repeat extraction 6X
Crude LPS-Receptor
Crude 1H
Centrifugation 33,000-151,000g >90% activity in aqueous top layer, sepharose 4B column, 0 05 M Tris, pH 7 0
DEAE column, elution 0 1 M phosphate buffer, pH 7 0, with continuous gradient, 0 1-1 0 M NaCl, Sephadex G-200 column, PO4 buffer, pH 7 5, 1H in void volume
Purified LPS-Receptor
Purified 1H
DEAE-Sephadex A-25 column elution 0 05 M Tris, pH 7 0, with continuous gradient, 0 05-0 5 M NaCl, Sucrose density gradient, 5%-25%, Sephadex G200 column, 0 05 M Tris, pH 7 0, receptor in void volume
Highly Purified LPS-Receptor

after every second water change. Following the final wash the stromata was collected by centrifugation, weighed and stored at -22°C until use.

Isolation and purification of the LPS receptor. The LPS-receptor was prepared as outlined in Figure 2 using a modification of the procedure of Springer et al. (15). A 50% aqueous stromata suspension was homogenized in a Waring blender and extracted overnight with two volumes of n-butyl alcohol at 4°C for 16 hours at pH 8.2. Four phases, resolved after centrifugation at 2000xg for 30 minutes, organic, lipid, aqueous and solid. The aqueous phase was re-extracted twice with n-butanol, once for 30 minutes and again overnight and then thoroughly dialyzed against several changes of 0.05 M Tris-HCl buffer (pH 7.0). An aqueous butanol extract low in LPS-receptor activity but high in IH inhibitor activity was obtained by shifting the pH of the butanol extraction from 8.2 to 5.3.

The dialyzed active crude butanol extract was centrifuged at 151,000 xg for 1-2 hours in a Spinco Model L2 preparative ultracentrifuge. Three phases resulted from the high speed centrifugation. Contrary to Springer's findings, the top aqueous layer possessed the highest LPS-receptor. After extensive dialysis, the aqueous top layer was applied to a 92 X 1.5 cm sepharose 4B column (Pharmacia Fine Chemicals, Piscataway, N.J.). The sepharose columns were washed with a 0.05 M Tris-HCl buffer at pH 7.0. Three milliliter fractions were collected and assayed for both LPS-receptor and IH inhibitor activities. The active sepharose fractions were pooled, concentrated tenfold by dialysis against 20% polyethylene-glycol in 0.05 M Tris-HCl buffer (pH 7.0) and applied to a 22.0 X 2.5 cm

DEAE-Saphadax A25 (Pharmacia Fine Chemicals, Piscataway, NJ) column After extensive washing of the column with the starting buffar, a linear sodium chloride gradient was initiated with 150 ml of 0 05 M pH 7 0 Tris-HCl buffer and 150 ml of 0 75 M, pH 7 5 Tris-HCl buffer Three milliliter fractions were collected and assayad for the two activities

Ether extraction Sometimes LPS-receptor or binding activity could not be localizad in tha aqueous butanol phase aftar shift in the pH during extraction The intermediate lipid phasa was then further extracted with equal volumes of ether (Mallanchrodt, St Louis, MO) Equal volumes of the lipid phase, suspanded in 0 05 M Tris-HCl buffer at a pH of 7 0 (1 2) and ather were vigorously mixed in a separatory funnel for 5-15 minutes. Aftar phase saparation the ether was removed from the aquaous and syrupy interphase layars by dialysis against phosphate buffared saline (pH 7 4) and from tha organic phasa by evaporation using a stream of nitrogen at 4°C Following evaporation, the residue remaining from the organic phase was reconstituted to its original volume with PBS and all phases were tested for LPS-receptor and IH inhibitor activities

Polyacrylamide gel electrophoresis (PAGE) Olsc polyacrylamide alactrophorasis was parformed using a modification of the method of Maurer (57) Extracts were appliad to 7 5% acrylamide gels and were electrophoresed in a non-reducing Tris-glycine buffer system, pH 8 6, for 45 minutes at 4°C. The gels were stained with 0 02% Coomassia blue containing 12 5% trichoracetic acid For soma studies, duplicate gals were run One gel was stainad as ahova with the remaining gel being sliced for analysis for LPS-recaptor and IH inhibitor activities

Lipopolysaccharide (LPS) preparations of Salmonella typhimurium LPS extracted by the Boivin (58) and Westphal (45) procedures were purchased from Difco Laboratories (Detroit, MI). Heated (100°C for 3 hours) and unheated LPS stock solutions (1.0 mg/ml PBS at pH 7.4) were stored at -22°C until used.

Antisera. Appropriate dilutions of Salmonella group B O-antiserum obtained from Baltimore Biological Laboratories (Cockeysville, MD) were made in 0.01 M EDTA GVB++. Hemagglutination (HA) titers of the sera ranged from 64 to 256.

For hemolytic assays, rabbit IgG antibodies to sheep erythrocyte stromata were obtained from Cordis Laboratories (Miami, FL). Stock solutions at a dilution of 1:100 in PBS (pH 7.4) were maintained until use at -22°C.

Treatment of erythrocytes with LPS. Freshly acquired erythrocytes from a group O, Rh positive adult were obtained and used in the coating and coating inhibition assays which were carried out as described by Springer et al. (14). For speed and economy, screening assays were assessed using a microtiter hemagglutination system. Briefly, the prodedure consisted of mixing equal volumes of either human or sheep erythrocytes at 2×10^8 cells/ml and dilutions of LPS for 45 minutes with shaking at 37°C. After extensive washing, the smallest amount of LPS which afforded maximal hemagglutination by subsequently added antiserum was determined. This dilution, defined as the optimal coating dose, was used in all subsequent hemagglutination-inhibition assays.

LPS-raceptor a[illegible] assay LPS-receptor activity was determined [illegible] measuring the ability of a material to inhibit LPS fixation to [illegible]rythrocytes The procedure in the coating HA-inhibition assay differed [illegible] that in the coating test in that dilutions of LPS binding material were added to equal volumes of an optimal coating dose of LPS and incubated with shaking for 30 minutes at 37°C Erythrocytes were addad, and HA titers datarmined as previously described In each assay, a control consisting of LPS and erythrocytes but no LPS-receptor material followed by tha subsequant addition of antiserum was includad

Isotonic buffer solutions employed in complement asseys The basic diluent for most hemolytic assays was the isotonic gelatin veronal buffer (GVB) described by Kabat and Mayer (55) which contained 0 00015 M $CaCl_2$, 0 0005 M $MgCl_2$, and 0 1% gelatin at pH 7 5 In some cases, gelatin veronal without $CaCl_2$ or $MgCl_2$, containing enough isotonic ethylanediaminetetra acetate (EDTA, pH 7 4) to bring the final concentration to either 0 01 M or 0 04 M, was employed These buffers were dasignated as 0 01 M EDTA-GVB and 0 04 M EDTA-GVB respactively In order to achieve maximum sensitivity, hemolytic assays involving individual complement components, and IH inhibitor asseys were performed using a low ionic strength gelatin-veronal prepared by mixing equal volumes of 5 0% glucose with gelatin-veronal buffar containing twice tha standard amount of $CaCl_2$ and $MgCl_2$ (DGVB)

Sensitized sheep erythrocytes (EA) Shaep erythrocytas at a concentration of 10^9 par ml in 0 01 M EDTA-GVB were mixed with an equal voluma of antibody to sheep stromata at a final dilution of 1-500 in

e buffer re was incubated with shaking for 30 minutes
and then at 0 for either 30 minutes or overnight The cells
ished twice and standardized to the desired concentration before

Complement (GPC) Fresh frozen guinea pig complement was obtained
rom Pel Freeze Laboratories (Rogers, AR) The serum was shipped in
dry ice and was stored at -70°C until use In some studies, aliquots
of GP serum were absorbed three times at 0°C with either untreated or
LPS treated sheep or human erythrocytes before use

Complement Components Guinea pig C1 and C2 were prepared by
methods described by Nelson et al (59) and Ruddy and Austin (50,61)
Individual lyophilized functionally pure guinea pig complement compo-
nents C3, C5, C6, C7, C8 and C9 were purchased from Cordis Laboratories
(Miami, FL)

Complement component intermediates For IH inhibitor assays and
complement consumption studies, sheep erythrocytes in the intermediate
state EAC$\overline{1}$, EAC$\overline{14}$, and EAC$\overline{142}$ were prepared by the methods of Borsos
and Rapp (62)

Determination of Tmax of EAC$\overline{142}$ The kinetics of the generation
of EAC$\overline{142}$ was determined by the Tmax procedure described by Borsos
et al (35)

IH inhibitor preparation Crude human erythrocyte stromata ex-
tracts, high in IH inhibitor activity, were isolated by a procedure
described by Hoffmann (50) The method is outlined in Figure 2
Briefly, the essential differences in Hoffmann s preparation of butanol

[illegible] cts high in IH inhibitor ac[illegible] and extracts high in LPS-receptor activity as defined by Springer are (1) stromata were prepared at pH of 6 0 - 7 0 without the addition of phenol, (2) crude washed stromata were suspended in equal volume of 0 005 M potassium phosphate buffer at a pH of 7 5 and extracted with n-butanol at a final concentration of 20% for 15 minutes, and, (3) after the first extraction the aqueous butanol phase was adjusted to an ionic strength of 0 15 by the addition of 3 0 M NaCl Butanol extraction of the adjusted material was repeated until a lipid phase could no longer be separated For further purification, concentrated material, active in IH activity, was subjected to gel filtration and DEAE chromatography

Treatment of sheep erythrocytes with partially purified IH inhibitor material Lipopolysaccharide coated and untreated sheep and human erythrocytes were treated with IH inhibitor material by the procedure described by Hoffmann (50) Equal volumes of erythrocytes at 10^8 cells/ml in OGVB and extracts of IH inhibitor diluted 1 10 in OGVB were mixed at 0°C The mixture was transferred to a 30°C water bath, incubated 30 minutes with shaking and was pelleted at 500xg for 10 minutes at 4°C The coated cells were washed two times, and standardized to the desired concentration in the appropriate buffer

IH inhibitor activity IH inhibitor activity was assessed using the EAC$\overline{14}2$ inactivation assay described by Hoffmann (50) Sheep erythrocytes in the intermediate state EAC$\overline{14}2$ at 10^8 /ml were mixed with an equal volume of IH inhibitor material diluted in OGVB The reaction mixtures were incubated at 30°C for 15 minutes with constant shaking,

after which, three volumes of guinea pig complement diluted 1 25 in 0 04 M EDTA-GVB were added The tubes were then incubated for 60 minutes at 37°C with shaking At the end of the incubation period, 10 volumes of ice-cold PBS was added to each reaction mixture The cells were pelleted at 500xg for 10 minutes at 4°C and the optical densities of the supernatant fluids were determined at a wave length of 414 nm

Chelators Stock solutions of disodium ethylenediaminetetra acetate (EDTA, Fisher Scientific Co., Fair Lawn, NJ) and ethyleneglycolbis (beta-amino ethyl ether) N[1],N tetraacetic acid (EGTA, Sigma Chemical Co., St. Louis, MO) were prepared as described by Fine et al. (35) The stock solutions were stored at 4°C and diluted to a final concentration of 200 mM before use. Magnesium EGTA was prepared as described by Fine et al (35)

Complement Consumption The ability of erythrocytes treated with LPS and/or IH inhibitor to consume complement was determined in reaction mixtures containing 0 1 ml of the treated cells (1×10^9 cells) or LPS and 0 9 ml of normal or absorbed guinea pig serum chelated with either EGTA or EDTA The mixtures were incubated with shaking 60 minutes at 37°C Following the incubation period, the cells were pelletnd at 500xg at 4°C for 10 minutes The supernatant fluids were reconstituted with magnesium and/or calcium and were analyzed for residual whole complement activity using a modification of the procedure as outlined by Kabat and Mayer (55)

Erythrocyte coating by LPS and its [illegible]ction Repeated titrations of Salmonella typhimurium LPS at concentrations ranging from 0 195 µg to 50 0 µg/ml as determined with polyvalent and homologous Salmonella antiserum, employing erythrocytes at 2 0 X 10^8 cells/ml were carried out The results of a representative experiment employing polyvalent antiserum are given in Table II It can be seen that heating the LPS enhanced the erythrocyte coating capacity to a remarkable extent Additionally, a maximum titer resulted when erythrocytes were exposed to at least 0 78 µg/ml of heated LPS Therefore, an optimal coating unit (µ) of heated LPS (defined as the reciprocal of the greatest dilution of LPS producing complete hemagglutination by either polyvalent or homologous antiserum) was taken as 0 78 µg/ml Assays employing homologous antiserum to heated LPS yielded lower optimal coating doses of 0 78 µg/ml and 1 56 µg/ml, depending on the age of the antiserum These results were identical for human and sheep erythrocytes LPS extracted by the Westphal procedure resulted in an optimal coating dose of 0 39 µg/ml as also determined with homologous antiserum

LPS receptor activity, as evaluated in these studies, was based on the ability of a given erythrocyte preparation to inhibit the complete fixation of an optimal coating unit of LPS onto either sheep or human

TABLE I

Determination of Optimal LPS Concentration
Used for Coating Human Erythrocytes

[illegible] (μg/ml)	Titers[a] unheated	heated[b]
50.0	80	ND
25.0	160	ND
12.5	80	ND
6.25	80	ND
3.125	80	160
1.563	80	160
0.781	40	160
0.390	20	80
0.195	10	40

[a] The reciprocal of the dilution of anti-LPS serum affording maximal hemagglutination

[b] Stock solutions of LPS (1.0 mg/ml PBS) were heated 100°C for three hours

erythrocytes. Table III, Column A summarizes the results obtained with several crude aqueous butanol preparations of erythrocyte stromata. These data indicate that the range of LPS-receptor concentrations or dilutions needed to yield optimal inhibition of LPS erythrocyte coating varied with the source, concentration and condition (solvent) of the erythrocyte stromata extraction procedure.

iH inhibitor activity of crude butanol extracts of human and sheep erythrocyte stromata. EAC$\overline{1}42$ inactivation by a crude erythrocyte stromal extract, assayed by the technique described in the section on materials and methods is shown in Figure 3. This procedure was used to determine the inhibitory potency of most extracts. Color controls for the presence of hemoglobin in the higher concentrations of crude preparations were necessary. The reciprocal of the dilution of a crude butanol preparation yielding greater than 50% inhibition of the lysis of EAC$\overline{1}42$ by C-EDTA are also shown in Table III. These results clearly indicate that LPS-receptor and iH inhibitor activities are contained in significant amounts in crude butanol stromal extracts obtained by either Springer's or Hoffmann's procedures. Of interest also, is that the potency of the two activities varied to the same extent.

A comparison of the chromatographic properties of the iH inhibitor and LPS-receptor from erythrocyte stromal extracts. The above data suggests that the LPS-receptor and IH inhibitor are either identical or closely related molecules, therefore, additional evidence to resolve this issue was sought. Human and sheep erythrocyte stromata were subjected to modifications of Springer's purification procedure as outlined

Table II

Comparison of L S-receptor and IH Inhibitor Activities of Several Crude Extracts of Erythrocyte Membrane

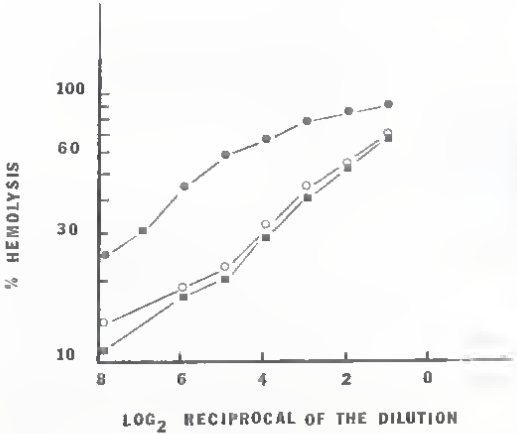
Crude Membrane Extract	Titers	
	A LPS-receptor[a]	B IH inhibitor[b]
Human cells extracted at pH 8 2 (Springer)		
Crude aqueous butanol phase	40	40
Crude aqueous phase		
high speed[c] top layer	320	320
high speed syrupy interphase	80	40
Human cells extracted at pH 7 5 (Hoffmann)		
Crude aqueous butanol phase	320	320
Sheep cells extracted at pH 8 2 (Springer)		
Crude aqueous butanol phase	0	0
Crude aqueous ether phase	0	0
Crude ether interphase	8	0
Crude ether organic phase	0	0

[a]The smallest amount (dilution) giving complete inhibition of LPS coating

[b]The reciprocal of the greatest dilution giving 50% inhibition of EAC142 lysis

[c]High speed extracts were obtained by the centrifugation of the crude aqueous butanol phase, 151,000xg for 2 hours

Figure 3. Inhibition of the hemolysis of EAC142 by C-EDTA in the presence of various concentrations of three crude phases resulting from the butanol extraction of human erythrocyte stromata prepared according to the procedure of Saringer. The open circles show the activity of the crude untreated butanol extracted stromata the closed circles show the inhibition associated with the top fraction obtained after the high speed centrifugation (40,000 rpm/2 hours) of the crude extracted stromata, the closed squares show IH inhibitor activity of interphase obtained after the high speed centrifugation of the extracted stromata


% HEMOLYSIS
100
60
30
10
8
6
4
2
0
LOG2 RECIPROCAL OF THE DILUTION

in Figure 2. Figure 4 is a gel filtration elution profile on sepharose 4B, of the crude high speed top layer obtained from the crude butanol human erythrocyte stromal preparation. Fractions were monitored at 220 and 280 nm and were assayed for LPS-receptor and IH inhibitor activities as previously described. Two peaks were observed with both activities eluting in the peak following the void volume. Close examination of the sepharose 4B profile indicates that there is a slight displacement of the IH inhibitor activity to the left of the LPS-receptor activity. This would suggest that perhaps the two activities may be different.

Further attempts to separate and purify the two activities were accomplished using ion exchange chromatography. The sepharose 4B active peaks were pooled, dialyzed against the starting Tris-HCl buffer, and applied to a DEAE-Sephadex column. Fractionation was accomplished with a linear NaCl gradient. A typical chromatogram of the partially purified material(s) is shown in Figure 5. LPS-receptor and IH inhibitor activities eluted in a relatively narrow peak at about 0.3 M NaCl, again with the IH inhibitor slightly preceding the LPS-receptor activity.

The recovery of the LPS-receptor and IH inhibitor activities following sepharose 4B and DEAE-Sephadex chromatography is presented in Table IV. It should be noted that gel filtration on sepharose 4B yielded only about onefold increase in the purity of both activities with recovery of only 52% of the LPS-receptor activity and 65% of the IH inhibitor activity. DEAE-Sephadex was shown to result in as much as a 19-fold increase in activity, but resulted in a recovery of 50% of the specific LPS-receptor activity but only one third or 31% of the

TABLE IV

Recovery of LPS-receptor and IH Inhibitor Activity from Extracts of Human Erythrocyte Stromata Following Sepharose 4B and DEAE-Sephadex Purification

Preparation	Volume (ml)	Concentration (A_{220}/ml)	Activity/ml LPSR[a]	Activity/ml IH_{50}[b]	Total Conc[c] (A_{220}/ml)	Total Activity LPSR	Total Activity IH_{50}	Specific[d] Activity LPSR	Specific[d] Activity IH_{50}	Yield[e] % LPSR	Yield[e] % IH_{50}	Purification[f] LPSR	Purification[f] IH_{50}
Sepharose 4B input (Crude high speed top layer conc)	4 5	479_2	320	320	2,156 4	1440	1440	0 67	0 67	—	—	—	—
Sepharose 4B recovery	47 0	18 5	16	20	871	752	940	0 052	1	[illegible]	[illegible]	[illegible]	[illegible]
DEAE-Sephadex recovery	6 1	10	128	80	61 1	780	488	12 80	7 0	[illegible]	31 1	19 1	11 8

[a] The smallest amount (dilution) giving maximum inhibition of LPS coating

[b] The reciprocal of the highest dilution giving 50% inhibition of EAC142 lysis

[c] Concentration

[d] Calculated by dividing the value for total activity by the values for total [illegible]

[e] Calculated by dividing the value for total activity after treatment, by the [illegible] treatment, multiplied by 100

[f] Calculated by dividing the value for each of the specific activities by the value for the initial specific [illegible]

Figure 3 A gel filtration profile of the butanol extracted human erythrocyte stromata prepared by the method of Springer. 4.5 ml of the extracted material were applied to a sepharose 4B column (92 x 1.5 cm) and chromatographed at 4°C with 0.05 M Tris buffer, pH 7.0. The optical density at 220 nm of the input material was 479 and 4.0 ml fractions were collected. Optical densities of the fractions at 220 are shown by the closed circles, the closed triangles indicate the elution pattern of the IH inhibitor activity, and the shaded bars represent the fractions having LPS-receptor activity

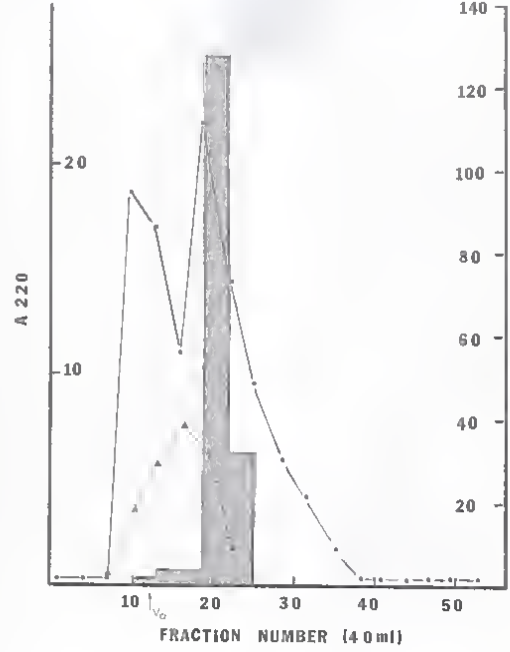
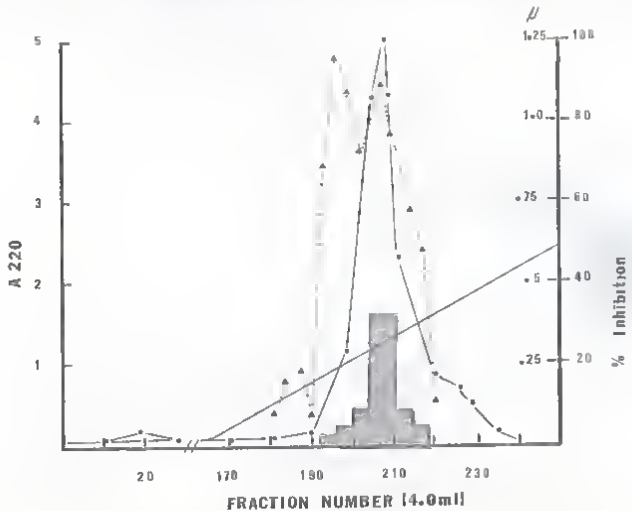

140
120
100
80
60
40
20
% Inhibition
20
10
A 220
10
Vo
20
30
40
50
FRACTION NUMBER (4 0 ml)

Figure 5 A DEAE-Sephadex A-25 chromatography profile of the sepharose 4B active fractions from human stromata extracts prepared by the method of Springer 6.1 ml of input material, with an optical density at 220 nm of 10.0 were applied to a ?? X ? cm column A linear sodium chloride gradient of 0.05-0.7M Tris buffer, pH 7.0 was applied and 4 ml fractions were collected Optical densities of the fractions at 220 nm are shown by the closed circles The closed triangles indicate the elution position of the fractions capable of inhibiting lysis of sheep EAC1$\overline{42}$ and the shaded bars represent the fractions having LPS-receptor activity

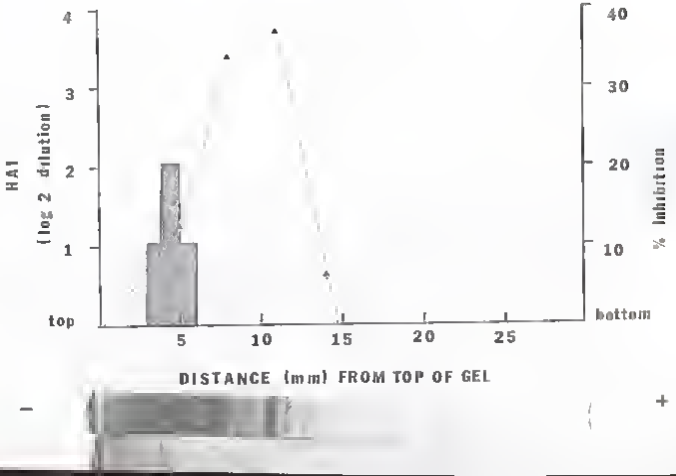

μ
1.25
1.0
.75
.5
.25
100
80
60
40
20
% Inhibition
A 220
5
4
3
2
1
20
170
190
210
230
FRACTION NUMBER (4.0ml)

IH inhibitor activity A comparison of the filtration and DEAE-Sephadex elution profiles (Figures 4 and 5) and the data in Table IV, revealing a 98% loss in the total mass but with a 10-fold increase in activity, indicate good purification of the two activities These data do, however, suggest further differences in the two activities by the differences in their respective recoveries following sepharose 4B and DEAE-Sephadex chromatography

The homogeneity of the active DEAE-Sephadex fractions was checked by electrophoresis on 7 5% polyacrylamide gels at pH 8 6 As shown in Figure 6, 6 - 12 protein bands were observed on the crude extracts with a sharp band and a diffused staining area localized at the top of the gel when partially purified DEAE-Sephadex fractions were electrophoresed Analysis of duplicate gels indicated that most of the LPS-receptor activity was localized in an area about 6 mm into the gels with the IH inhibitor activity spread over a fairly large area at the top of the gel with a peak of activity at about 11 0 mm (Figure 6)

IH inhibitor and LPS-receptor activities of sheep erythrocyte stromata The aqueous phase of butanol extracted sheep erythrocyte stromata, prepared by Springer's extraction procedure at pH 8 2 and at pH 5 3, had neither detectable LPS receptor nor IH inhibitor activities LPS-receptor activity but no IH inhibitor was, however, observed in an ether soluble fraction of the n-butanol lipid phase as was shown in Table III

Figure 6. Correlation between the distribution of LPS-receptor and IH inhibitor activities from extracts of human erythrocyte stromata when subjected to polyacrylamide disc gel electrophoresis. Extracts were applied to duplicate 7.5% gels and were electrophoresed in a non-reducing Tris glycine buffer, pH 8.6 for 45 minutes at 4°C. After electrophoresis, one gel was stained for protein with Coomassie blue and the other was cut into suitable segments which were eluted and analyzed for IH inhibitor and LPS-receptor activities. The closed triangles indicate the elution position of the IH inhibitor and the shaded bars represent the elution profile of the LPS-receptor activity


HAI
(log 2 dilution)
4
3
2
1
top
% inhibition
40
30
20
10
bottom
5
10
15
20
25
DISTANCE (mm) FROM TOP OF GEL
–
+

A significant difference in the two activities was evident when it was serendipitously observed that a shift in the pH from 8.2 to 5.3 in the butanol extraction step of the crude erythrocyte stromata resulted in a preparation consisting of little or no detectable LPS-receptor activity, but high in IH inhibitor activity. The elution profile of the IH inhibitor activity following ion exchange chromatography on DEAE-Sephadex of the partially purified material, as shown in Figure 7, was similar to that observed for material extracted at pH 8.2. LPS-receptor activity assays were not carried out on the remaining butanol phases because these materials had been discarded before the impact of these observations were realized.

A shift in the pH to 5.3 of a crude butanol extract of erythrocyte stromata obtained at a pH of 8.2 affected neither the LPS-raceptor nor IH inhibitor activities. This suggested the possibility that the LPS-receptor activity of the material extracted at a pH of 5.3 was not destroyed but was probably redistributed into another phase. Stromal extractions were carried out at pH 5.3 employing smaller volumes of erythrocytes stromata, using Springer's procedure, in an attempt to localize the LPS-receptor activity. As shown in Table V, some IH inhibitor activity was observed in the crude aqueous butanol phase when 10.0 ml of packed stromata were extracted. There was no detectable LPS-receptor in this butanol layer. The lipid phase mixed with 10 volumes of PBS was further subjected to an ether extraction resulting in four layers a pellet, an aqueous layer, lipid interphase, and an organic layer. All of the LPS-receptor activity was recovered in the organic ether layer

TABLE V

Recovery of LPS-receptor and IH Inhibitor Activity of Human Erythrocyte Stromata Extracted at pH 5.3 Using the Procedure of Spri[illegible]

Preparation	Quantity	Total Concentration (A_{220}/ml)	Total Activity		Specific Activity[d]	
			LPSR[a]	IH_{50}[b]	LPSR	IH_{50}
Aqueous butanol	16.0 ml	15.0	0	32.0	0	[illegible]
Organic butanol	23.0 ml	ND[c]	0	0		
Lipid	1.2 g	ND[c]				
Aqueous ether	5.0	6.32	0	160	[illegible]	
Lipid interphase	4.5	ND	0	144	[illegible]	
Organic ether	12.0	313.2	192	[illegible]	[illegible]	

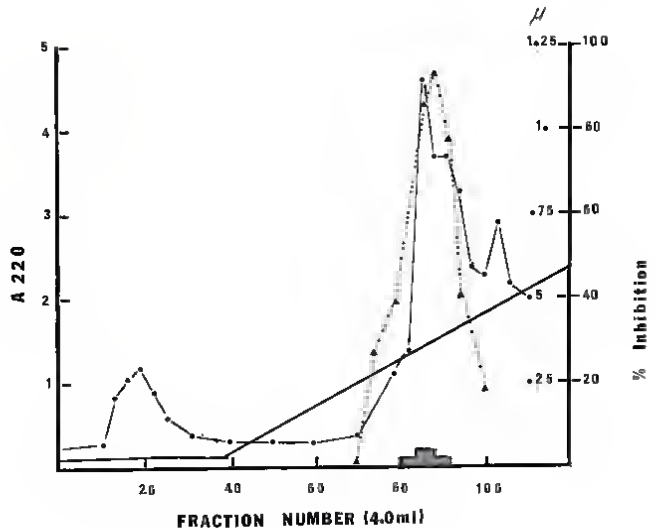
[a]The smallest amount (dilution) giving maximum inhibition of LPS activity

[b]The reciprocal of the highest dilution giving 50% inhibition of EACT2[a] lysis

[c]Not determined, due to high levels of particulate matter

[d]Calculated by dividing the value for total activity by the value for [illegible]

Figure 7. A DEAE-Sephadex A-25 chromatography profile of the butanol extracted human erythrocyte stromata prepared at pH 5.3 by the method of Springer. 40.0 ml of the input material with an optical density at 220 nm of 9.9 were applied to a 22 X 2.5 cm column. 4.0 ml fractions were eluted with a linear sodium chloride gradient of 0.05-0.75 M, Tris buffer at pH 7.0. Optical densities of the fractions at 220 nm are shown by the closed circles. The closed triangles indicate the elution position of the fractions capable of inhibiting lysis of sheep EAC1$\bar{4}$2 and the shaded bars represent the fractions having LPS-receptor activity.


μ
1.25
1.
.75
.5
.25
100
80
60
40
20
% Inhibition
A 220
5
4
3
2
1
20
40
60
80
100
FRACTION NUMBER (4.0ml)

[illegible] aqueous and [illegible] interphases. A comparison of the purification tables (IV and VI) [illegible] crude extracts obtained at pH 8.2 and pH 5.3 indicates that, as was observed at pH 8.2, extracts obtained at pH 5.3 and subjected to DEAE-Sephadex chromatography resulted in a substantially greater loss of total mass as estimated by the adsorbance at 220 nm and the yield of IH inhibitor activity.

Treatment of Springer's crude butanol extracts with sheep erythrocytes. To further establish that the two activities are distinctly different, equal volumes of sheep erythrocytes at either 10^8 or 10^9 cells/ml were mixed with equal volumes of a butanol high speed top layer extract prepared according to the procedure of Springer. This extract had an initial LPS-receptor titer of 128 and a IH_{50} inhibitor titer greater than 80. Control tubes consisting of equal volumes of buffer and the extracts were also prepared. All tubes were mixed at 30°C for 30 minutes with shaking. The cells were pelleted by centrifugation and the supernatant fluids along with the buffer controls were diluted and assayed for LPS-receptor and IH inhibitor activities. As can be seen in Figure 8, the IH inhibitor activity was reduced substantially when extracts were treated with 10^9 cells/ml. In contrast, the LPS-receptor activity remained constant when the cell treated and buffer control supernatants fluids were compared.

The biological consequence of the IH inhibitor and LPS-receptor on the erythrocyte membrane. The data in the previous section indicated two essential points. First, the membrane of the human erythrocyte,

Figure 6 Treatment of partial purified [illegible] erythrocyte stromata prepared by [illegible] Springer. Either 10^9 sheep erythrocytes [illegible] fer were mixed with an equal volumes of the [illegible] tracted material. Following a 30 minute incubation period at 37°C, the cell suspension was pelleted and all supernatant fluids were assayed for LPS-receptor and IH inhibitor activities. The hatched bars represent the sheep erythrocyte treated extracts, and the opened bars the buffer treated extracts.

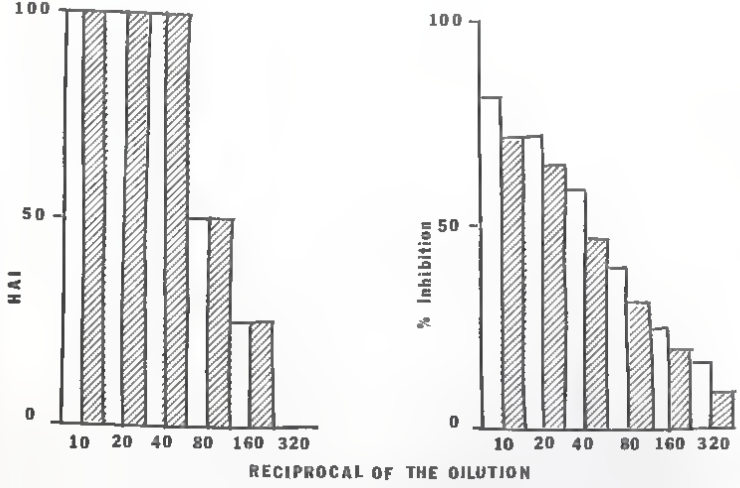

100
50
0
HAI
10 20 40 80 160 320
100
50
0
% Inhibition
10 20 40 80 160 320
RECIPROCAL OF THE DILUTION

TABLE VI

Recovery of IH Inhibitor and LPS-receptor Activities
From Erythrocyte Stromata Extracted at pH 5.3 by the Procedure
of Springer and Subjected to DEAE-Sephadex Chromatography

Preparation	Volume (ml)	Concentration (A_{220}/ml)	Activity/ml		Total Conc.[c] (A_{220}/ml)	Total Activity		Specific Activity		Yield %		Purification	
			LPSR[a]	IH_{50}[b]		LPSR	IH_{50}	LPSR	IH_{50}	LPSR	IH_{50}	LPSR	IH_{50}
DEAE-Sephadex Input	40	9.89	4	640	395.6	160	25600	0.4	64.7	-	--		
DEAE-Sephadex	5	13.74	4	160	68.7	20	800	0.29	11.6	12.5	3	1.4	5.6

[a] The smallest amount (dilution) giving maximum inhibition of LPS coating

[b] The reciprocal of the highest dilution giving 50% inhibition of EAC$\overline{142}$ lysis

[c] Concentration

[d] Calculated by dividing the value for total activity by the values for total concentration

[e] Calculated by dividing the value for total activity after treatment, by the value for the total activity before treatment, multiplied by 100

[f] Calculated by dividing the value for each of the specific activities by the value for the initial specific activity

which [illegible]ly resistant to complement mediated lysis, possesses at least two distinctly different molecules with biologically opposing properties lipoglycoproteins with a high affinity for lipopoly saccharides which are potent activators of the complement system, and a class of molecules shown to be potent inactivators of complement Second, sheep cells which are normally extremely sensitive to immune lysis have been shown to be devoid of the IH inhibitor but possess molecules with an affinity for LPS which are confined to the lipid moiety of the membrane

As shown in Figure 9, the interaction of free LPS (extracted by both the Boivin and Westphal procedures) with guinea pig serum which had been absorbed with sheep E coated with LPS (E-LPS), resulted in a substantial consumption of complement Additionally, it can be seen that LPS (Boivin) appeared to be a much more efficient activator of the alternative complement pathway compared to LPS extracted by the Westphal procedure

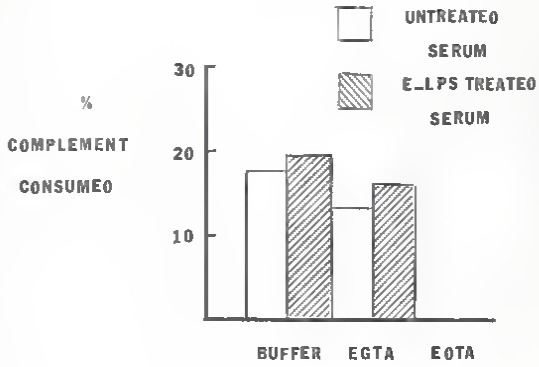
LPS (extracted by both procedures) coated onto the surfaces of sheep erythrocytes showed a similar profile (Table VII), except erythrocytes coated with LPS extracted by the Westphal procedure were far more efficient activators of complement in the absence of natural antibodies to LPS. It was of interest, therefore, to determine if LPS on the surface of sheep erythrocytes, in the presence of the IH inhibitor and E-LPS absorbed guinea pig serum, would alter the complement consumption profile of E-LPS To explore this possibility, erythrocytes were coated with LPS and IH inhibitor then reacted with guinea pig serum (absorbed

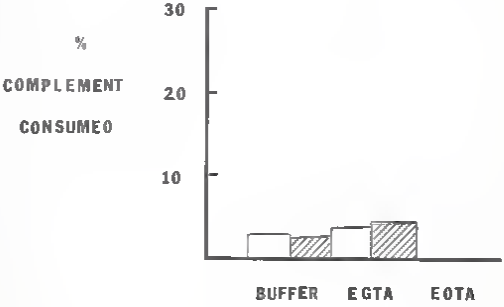
TABLE

of Total Complement [illegible] Absorbed
or E-LPS Absorbed GP Serum
by Untreated and LPS Coated Sheep Erythrocytes

[illegible] on	Percent Consumed	
	E absorbed serum	E-LPS absorbed serum
	--	--
LPS Westphal	21 3	16 89
LPS Boivin	47 9	6 38

Figure 9 Consumption of complement in normal and E-LPS treated guinea pig serum by fluid phase Salmonella typhimurium LPS extracted by the Boivin and Westphal procedures EDTA, EGTA, and saline treated sera were treated with the LPS preparations one hour at 37°C, then sedimented, after which the CH50 titers were determined in the magnesium and calcium reconstituted sera. The top figure represents the profile of the Boivin treated serum and the lower figure, the Wesphal treated serum

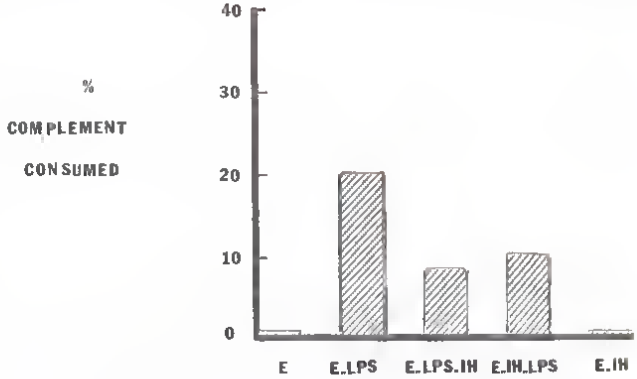

UNTREATED SERUM
E_LPS TREATED SERUM
%
COMPLEMENT
CONSUMED
30
20
10
BUFFER
EGTA
EDTA


%
COMPLEMENT
CONSUMED
30
20
10
BUFFER
EGTA
EDTA

[illegible] cells were prepared [illegible] was coated with LPS only (E-LPS), a second group was coated with [illegible] first, then was treated with the IH inhibitor (E-LPS-IH), a third [illegible] was treated with IH inhibitor first followed by the LPS-receptor (E [illegible] LPS), a fourth group was treated with IH inhibitor only (E-IH), and, a fifth group of cells (E) was treated with PBS under the same conditions and served as the control The efficiency of the LPS coating procedure in the presence and absence of the IH inhibitor was evaluated by assaying the five groups of cells and their respective supernatant fluids for LPS activity, employing the hemagglutination assay (described in Materials and Methods) as determined with antiserum to Salmonella typhimurium group B The results of these assays indicated that all of the LPS treated cells adsorbed equal quantities of LPS in the presence and absence of the IH inhibitor The complement consumption profiles of the five groups of erythrocytes treated with E-LPS Westphal absorbed guinea pig serum are presented in Figure 10 It can be seen that the presence of IH inhibitor on the cell caused about 50% reduction in the LPS mediated hemolytic action of complement Of particular interest was the fate of the cells when complement was treated with IH - LPS coated erythrocytes, significantly less cells were lysed This was in contrast to the case where complement was mixed with E-LPS and the cells were completely lysed These results suggested the possibility that complement activation had taken place, but that cells were protected from lysis by the presence of IH inhibitor on the membrane

Table 14

Consumption of complement in E-LPS westphal absorbed guinea pig serum by LPS and/or IH inhibitor coated sheep erythrocytes. One tenth ml containing 10^8 sheep erythrocytes (E) either untreated or treated with LPS (E-LPS), IH inhibitor (E-IH) or LPS and IH inhibitor (E-LPS-IH) were incubated with 0.9 ml of guinea pig serum at 37°C for 1 hr. Residual complement hemolytic activities were assayed and the % of the available complement consumed was calculated


%
COMPLEMENT
CONSUMED
40
30
20
10
0
E
E.LPS
E.LPS.IH
E.IH.LPS
E.IH

DISCUSSION

The experiments reported here have demonstrated that extracts from human erythrocyte membranes possessing LPS-receptor activity obtained by the method of Springer et al (15) were also capable of inhibiting complement mediated lysis The anticomplementary activity of these extracts was demonstrated to share many of the properties of the IH inhibitor previously described by Hoffmann (50)

Data are presented which strongly suggest that the two biologic activities are closely associated, but separable Evidence for this was provided by the results of five different experimental approaches in the analysis of Springer human erythrocyte stromal extracts The first was based on the chromatographic properties on sepharose 4B and DEAE-Sephadex where slight differences between the elution patterns and recoveries of the two activities were observed The second piece of evidence came from the electrophoretic profile of the crude and partially purified extracts on 7 5% polyacrylamide gels under non-reducing conditions- The IH inhibitor activity was shown to cover a fairly large area at the top third of the gel with the LPS-receptor activity being localized in a narrow, single band with a peak of activity near the top of the gel A third item of evidence was based on the redistribution and separation of the two activities into different phases when the pH during the crude stromal butanol extraction procedure

shifted from S 2 to S 3 The fourth approach, based on the high affinity of the IH inhibitor for the membranes of sheep erythrocytes, demonstrated that the IH inhibitor activity was partially removed leaving the LPS-receptor activity unchanged by the treatment of the stromal extracts with sheep erythrocytes Finally, evidence was presented indicating that the binding specificity of sheep erythrocytes for LPS of gram negative bacteria is localized in a lipid moiety of the crude stromal extracts and is free of all detectable IH inhibitor activity

It should be emphasized, however, that these experiments cannot exclude the possibility that both activities may be associated on the same macromolecule with the differences reported here being a consequence of experimental manipulation. That the two activities may be a function of a single macromolecule is certainly a major possibility Springer et al-(16) in assessing the chemical and physical properties of a homogenous preparation of the LPS-receptor, observed that both citraconylation and dissociating polyacryamide gel electrophoresis under standard conditions yielded two fragments, one of which absorbed significantly only at 230 nm Decitraconylation of the citraconylated fragment restored high LPS-receptor activity to only one of the fragments These studies are only suggestive and do not permit a decision on whether the activities are on the same molecule however

In contrast, the data obtained from sheep erythrocytes which completely lack IH inhibitor, but which possess LPS-receptor activity, would support the finding that the two macromolecules may be distinctly different However, the evidence would suggest that the LPS-receptors

on sheep cells differ from those observed on human cells, since they are confined to the lipid moiety

Good purification of the LPS-receptor and IH inhibitor activities following DEAE-Sephadex chromatography is indicated by the quantitative data tables These results would suggest, however, that as a preparative purification step it should be modified to encourage higher yields of the two activities

The observation that sheep erythrocyte membranes possess molecules with receptor specificity for LPS is not surprising for it has long been known that sheep cells could be modified by the presence of LPS of gram negative bacteria and that these modified cells are readily lysed in the presence of homologous antiserum to LPS and complement (44) In contrast, hemolysis was not observed when LPS treated human erythrocytes were treated under the same conditions This raises the possibility that human erythrocytes, a natural source of the IH inhibitor even when treated with LPS, are extremely resistant to LPS mediated lysis because of the presence of inhibitor molecules.

The findings reported here generally agree with those of Phillips et al (46) indicating that LPS treated sheep erythrocytes can activate the complement system in the presence of natural antibodies to the LPS. In contrast to their results, however, erythrocytes coated with a preparation of LPS extracted by the procedure of Westphal were shown to be capable of activating the complement system in the absence of natural antibodies to LPS Additionally, fluid phase LPS extracted by the Bolvin method (LPS-bolvin) was shown to be a more effective activator of the

complement system than LPS extracted by the Westphal procedure (LPS-westphal) in the presence and absence of natural antibodies to LPS However, once LPS extracted by the Boivin becomes cell associated, its capacity to activate the complement system in the absence of natural antibodies is greatly diminished This is significant because LPS activation of the complement system by an antibody independent mechanism requires either an exposed lipid A moiety or polysaccharide core (8,19) This would suggest then that the orientation of LPS-boivin on the membrane may be different from that of LPS-westphal, resulting in the masking of active sites necessary for the activation of complement

The fact that different preparations of LPS from the same species, when coated onto the surface of sheep erythrocytes, activated the complement system to different degrees and by different pathways, depending on the presence or absence of natural antibodies to LPS, introduces the possibility that the LPS activation of compiement may require substances other than the LPS molecule alone This especially appears to be true since LPS extracted by the Westphal procedure, which was shown to activate compiement in the presence and absence of natural antibody to LPS, is known to contain less protein and lipoproteins than LPS extracted by the Boivin procedure

Placing the IH inhibitor on LPS treated sheep erythrocytes reduced the ability of erythrocytes to consume complement The fact that the cell is protected even when compiement is activated suggests that the IH inhibitor may occupy specific sites on the red cell membrane rendering it resistant to immune lysis, but also leaving it available

to partially inhibit immune lysis. This may be due either to the masking of LPS-receptors resulting in less LPS uptake (demonstrated not to be the case here), or the prevention of C3b fixation to the cell membrane thus blocking the initiation of the membrane attack system of serum complement. An analogous site on human erythrocytes is already occupied by the IH inhibitor rendering this cell naturally immune to LPS mediated lysis.

The findings presented here have led us to hypothesize that a necessary criterion for resistance to LPS induced complement mediated lysis would be the localization of LPS-receptor and IH inhibitor molecules on the same membrane. This would imply that any red cell devoid of IH inhibitor molecules would be far more susceptible to the cytolytic action of LPS activated complement.

LPS of gram negative bacteria are potent activators of the complement system. It is of great clinical interest, therefore, that substances on the surface of erythrocytes which bind LPS are found closely associated with materials capable of inhibiting complement mediated lysis. The symptoms of several infectious diseases, such as typhoid fever, have been observed to include very intensive erythrophagocytic activity by macrophages of lymph nodes. The consequence of this observation could have great clinical importance. Erythrocytes coated with LPS, in contact with serum complement, would naturally lead to activation of the complement system followed by increased phagocytosis with minimal cytolysis. This would lead naturally to an amplification of the activation of the complement cascade resulting in either clearance or a heightened inflammatory response.

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Gloria Jean Jackson, [illegible] daughter of [illegible] and Mrs. Mamie McKnight, was the second of [illegible] attended and was graduated May 27, 1957, [illegible] of my class at Boylan Haven, a private school for girls [illegible] at that time in Jacksonville, Florida

I later attended Bennett College, majoring in premedicine with a minor in psychology, graduating June of 1961. Lack of funds prevented entry into medical school, therefore, I was employed as a technician at University of Florida for one year. I later entered the University of Kansas at Lawrence, Kansas to pursue a master's degree in Microbiology specializing in microbial physiology. After a year and a half I completed the investigative requirements for the master's degree and was employed as a research technician in the Department of Microbiology, where I served as an assistant to a Microbial Geneticist. In October 1965 I was married to Virgil Lawrence Jackson, following which we relocated to Chicago, Illinois.

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Following a legal separation, I returned with my son, Jacques, to Gainesville to pursue a doctorate at the University of Florida, majoring in Microbiology and specializing in Immunology. I am presently employed at Abbott Labs of North Chicago as a product manager in research and development. In addition to immunology, I enjoy tennis, chess, bridge, skiing, and sailing.

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